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(54) Title: INJECTABLE COLLAGEN-BASED SYSTEM FOR DELIVERY OF CELLS OR THERAPEUTIC AGENTS

(57) Abstract: Disclosed herein is a method for delivering cells or a therapeutic agent to a tissue site, the method involving (a) combining the cells or therapeutic agent with a soluble collagen to form a cell-collagen or therapeutic agent-collagen solution; and (b) administering the solution to the tissue site, whereby, upon contact with the tissue, the solution is converted to a gel. Also disclosed herein are the use of this method for treating tissue defects, and useful cell-collagen and therapeutic agent-collagen solutions.

INJECTABLE COLLAGEN-BASED SYSTEM FOR DELIVERY OF CELLS OR THERAPEUTIC AGENTS

Background of the Invention

In general, this invention relates to a collagen delivery system to administer a therapeutic agent such as a cell preparation, a growth factor, a steroid, an antibiotic, or a regulator of angiogenesis or cell proliferation.

Numerous properties of collagen favor its use as a biological delivery agent (Simpson, Biomaterials in Reconstructive Surgery, In *Collagen as a Biomaterial*, C.V. Mosby Co.,Ch. 11, pages 109-117, 1983; DeVore, Collagen as an Opthalmic Biomaterial, In *Encyclopedic Handbook of Biomaterials & Bioengineering, Part B, Applications*, Marcel Dekker, Inc., New York, Ch. 45, 1995; Silver and Garg, Collagen: Characterization, Processing and Medical Applications, In *Handbook of Biodegradable Polymers*, Eds. Domb, Kost, and Wiseman, Harwood Academic Publishing, Australia, Ch. 17).

For example, one can design collagen products in which the time required for resorption ranges from a few minutes to months. This rate of collagen resorption is controlled by the form of collagen extracted or prepared and by the degree of collagen crosslinking. In particular, collagen molecules with stabilized, reducible, intramolecular and intermolecular crosslinks take the form of insoluble, condensed collagen fibrils that are resorbed slowly *in vivo*. In contrast, collagen molecules with few, if any, crosslinks are soluble in aqueous salt, acidic solutions, and basic solutions, and are quickly resorbed. Therefore, one can prepare collagen in various forms or can chemically modify collagen to obtain formulations of the desired resorption time while still retaining collagen's hydrophilic and non-immunogenic properties.

The ability to specifically deliver a compound to a particular site in the human body is a desirable goal in many areas of medicine. Such delivery (especially by injection) is particularly beneficial, for example, to accurately administer a sufficient dose to the site of interest while, at the same time, minimizing exposure to surrounding tissues. Site specific delivery is beneficial, for example, to enhance bone repair (Miyamoto and Takaoka, Ann. Chir. Gynaecol. Suppl. 207:69-75, 1993), cartilage repair (Buckwalter and Mankin, Instr. Course Lect. 47: 487-504, 1998), tendon repair (Young et al., J. Orthop. Res. 16: 406-413, 1998), wound healing (Rao, J. Biomater. Sci. Polymer Edn. 7: 623-645, 1995), and peripheral nerve regeneration (Seckel et al., J. Neurosci. Res. 40: 318-324, 1995), as well as to prevent local infection (Wachol et al., Biomaterials 17: 1733-1738, 1996; Gebhardt and Kaufman, J. Ocul. Pharmacol. Ther. 11: 319-327, 1995), cellular proliferation (Kay et al., Ophthalmic Surg. 17: 796-801,1996), and local tumor growth (Davidson et al., J. Surg. Res. 58: 618-624, 1995)

The remodeling of the adult skeleton involves the ongoing processes of bone formation and resorption as well as the regulation of serum calcium. Following bone injury, significant numbers of osteoprogenitor cells can be mobilized at the defect site to produce large quantities of bone matrix. In addition, mesenchymal cells migrate from the marrow and differentiate into chondroblasts that produce a cartilagenous extracellular matrix, subsequently replaced by bone. These processes are controlled, at least in part, by the large number of growth factors present in the bone matrix after injury (Wozney and Rosen, Handbook of Experimental Pharmacology, In *Physiology and Pharmacology of Bone*, Eds. Mundy and Martin, Springer Verlag, Berlin, Vol. 107, 1993).

The cartilage injury response differs from other tissues because of

the relative avascularity of cartilage and the limited chondrocyte mobility and proliferation in cartilage (Newman, Am. J. Sports Med. 20: 309-324, 1998). In other tissues, by contrast, the initial stage of wound healing is characterized by the movement of intravascular components, such as plasma and blood proteins, to the extravascular area (Peacock, In *Wound Repair*, Ed. Peacock, WB Saunders Co, Philadelphia, PA, pages 491-492, 1984). Neutrophils and macrophages then migrate to the injury site, functioning to prevent infection and promote fibroblast migration. Subsequent phases of wound healing include fibroblast secretion of collagen, collagen stabilization, angiogenesis, and wound closure (Costa et al., Opth. Surgery 24: 152-170, 1993).

Summary of the Invention

In general, the invention features a method for delivering a therapeutic agent to a tissue site, the method involving: (a) combining the therapeutic agent with a soluble collagen to form a therapeutic agent-collagen solution; and

(b) administering the solution to the tissue site whereby, upon contact with the tissue, the solution is converted to a gel and the therapeutic agent is delivered to the site.

In preferred embodiments, the tissue is mammalian, more preferably human, the solution is administered by injection, the tissue site is bone, cartilage, meniscus, tendon, ligament, or dermis, the tissue site has a defect, and the defect is treated by delivery of the therapeutic agent to the tissue site.

In other preferred embodiments, upon administration of the solution, the solution is converted to a gel within 180 seconds, more preferably, within 120 seconds, most preferably, within 90 seconds, and the solution has a fibrillar component, preferably, the fibrillar component is at a concentration of between

0.01-2.0%, more preferably, 0.2-1.2% fibrillar collagen solids (w/v).

The duration of delivery of the therapeutic agent is extended by increasing the intramolecular or intermolecular crosslinking of the collagen in the solution (preferably, by adding a fibrillar component to the solution, or induced chemically or by UV radiation).

Preferably, the therapeutic agent is a growth factor, a steroid, an antibiotic, an angiogenic agent, an inhibitor of angiogenesis, or an inhibitor of cell proliferation, and the therapeutic agent-collagen solution is at approximately pH 7.0.

In a second and related aspect, the invention features a therapeutic agent-collagen solution, which, upon contact with a tissue, is converted to a collagen gel.

In preferred embodiments of this second aspect, the solution is converted to a gel upon contact with mammalian tissue, more preferably, human tissue, the therapeutic agent-collagen solution is injectable, the therapeutic agent-collagen solution is converted to a collagen gel within 180 seconds, more preferably, within 120 seconds, and, most preferably, within 90 seconds, and the therapeutic agent-collagen solution further comprises a fibrillar component, preferably, the fibrillar component is at a concentration of between 0.01-2.0%, more preferably, 0.2-1.2% fibrillar collagen solids (w/v).

In other preferred embodiments, the therapeutic agent is crosslinked to the collagen in the solution or the collagen is intramolecularly or intermolecularly crosslinked in the solution (preferably, the crosslinking is induced chemically by UV radiation).

Preferably, therapeutic agent is a growth factor, a steroid, an antibiotic, an angiogenic agent, an inhibitor of angiogenesis, or an inhibitor of cell proliferation, and the solution is at approximately pH 7.0.

The invention also includes a method for delivering cells to a tissue site, the method involving: (a) combining the cells with a soluble collagen to form a cell-collagen solution; and (b) administering the solution to the tissue site, whereby, upon contact with the tissue, the solution is converted to a gel and the cells are delivered to the defect.

In preferred embodiments of this third aspect of the invention, the tissue is mammalian, preferably human, the cells are stem cells, preferably mesenchymal stem cells, the solution is administered by injection, the tissue site is bone, cartilage, meniscus, tendon, ligament, or dermis, the tissue site has a defect, and the defect is treated by delivery of the cells to the tissue site.

In other preferred embodiments, upon administration of the solution, the solution is converted to a gel within 180 seconds, more preferably, within 120 seconds, and, most preferably, within 90 seconds, the solution has a fibrillar component, preferably, the fibrillar component is at a concentration of between 0.01-2.0%, more preferably, 0.2-1.2% fibrillar collagen solids (w/v), and the cell-collagen solution is at approximately pH 7.0.

The duration of delivery of the cells is extended by crosslinking the cells to the collagen in the solution (preferably, the crosslinking is induced chemically or by UV radiation) or by increasing the intramolecular or intermolecular crosslinking of the collagen in the solution (preferably, by adding a fibrillar component to the solution, or induced chemically or by UV radiation).

In a fourth and related aspect, the invention features a cell-collagen solution, whereby, upon contact with a tissue, the solution is converted to a collagen gel.

In preferred embodiments of this second aspect, the cell-collagen solution is converted to a gel upon contact with mammalian tissue, more

preferably, human tissue, the cells are stem cells, more preferably mesenchymal stem cells, the cell-collagen solution is injectable, the cell-collagen solution is converted to a collagen gel within 180 seconds, more preferably, within 120 seconds, most preferably, within 90 seconds, and the therapeutic agent-collagen solution further comprises a fibrillar component, preferably, the fibrillar component is at a concentration of between 0.01-2.0%, more preferably, 0.2-1.2% fibrillar collagen solids (w/v), and the solution is at approximately pH 7.0.

By "tissue" is meant an aggregation of similarly specialized cells in an organism, preferably, mammalian, and, most preferably, human, where the cells are exposed to the organism's extracellular fluid, and are united in performance of a function within an organism.

By "therapeutic agent" is meant a substance that is administered to improve the condition of a tissue following injury or as a result of disease or degeneration.

By "fibrillar component" is meant an insoluble fibrillar collagen component wherein the collagen molecules interact in a quarter-stagger array to form microfibrils which themselves aggregate by side-to-side and end-to-end association to form stabilized collagen fibrils. The fibrillar component exhibits a collagen solid content ranging from about 0.1-2.0% (w/v) fibrillar collagen solids.

By "fibrillar collagen solids" is meant the dry collagen solid content of the fibrillar component in terms of percent fibrillar solids. Prior to addition to a soluble collagen formulation, the collagen solids are suspended in solution at a concentration ranging from 10-100 mg/ml, preferably 40 mg/ml. The collagen solid suspension is then added to the soluble collagen formulation for

a final collagen solid concentration of 0.1-2.0%, or 1-20 mg/ml.

The present invention provides a number of advantages. For example, the collagen delivery systems described herein are biocompatible, readily available, biodegradable, and stable in solution at neutral pH. The ability to manipulate the collagen in solution form allows for the homogeneous dispersion of the therapeutic agents and the injectable administration of these agents through a fine gauge needle (e.g., a 30 gauge needle). In addition to the ease of application, injectable delivery also allows access to the administration site while minimizing invasive injury to surrounding tissues. The density of the collagen solution is sufficient to fill a bone defect or other specific delivery site and remain in place until gelation and fibril formation occurs, as well as to maintain the active therapeutic agent in the injection site for a period of time sufficient to promote the effect.

The presently claimed collagen delivery system also has the advantage of allowing for the adjustment of therapeutic agent concentrations to achieve the desired dose. Furthermore, the crosslinking of molecules in the therapeutic agent-collagen formulation can be adjusted to extend the duration of therapeutic agent release. After polymerization, the injectable collagen delivery system also provides a scaffold-like structure to support new tissue growth.

In addition to the above, the collagen delivery system also has the advantage of providing local delivery. Thus, when the therapeutic agent has toxic side effects, e.g., chemotherapeutic agents, administration to a specific injection site minimizes exposure to surrounding tissues and dramatically reduces the undesirable, unnecessary side effects caused to the surrounding tissues or the body as a whole, while facilitating delivery of potent doses to the targeted cells. The local delivery system further has the advantage of

minimizing the total amount of therapeutic agent required, and thus the cost of treatment.

Other features and advantages of the invention will be apparent from the detailed description thereof and from the claims.

Description of the Drawing

FIGS. 1A (4X magnification) and 1B (20X magnification) are photographs of an MSC-collagen gel maintained in culture for 7 days and stained with toluidine blue. No extracellular matrix formed.

FIGS. 2A (4X magnification) and 2B (20X magnification) are photographs of an MSC-collagen gel, cultured *in vitro* for 7 days, implanted subcutaneously in nude mice for 3 weeks, and then removed and stained with toluidine blue. An extracellular matrix with chondrocyte-like cells was observed as well as chondrocyte hypertrophy and endochondral bone formation.

FIG. 3 (20X magnification) is a photograph of an MSC-collagen gel, initially injected subcutaneously in nude mice as a solution which subsequently polymerized. The gel was removed 6 weeks post-injection and stained with toluidine blue. Pronounced extracellular matrix formation was observed, including chondrogenesis, osteogenesis, and the formation of openings resembling marrow space.

FIGS. 4A-4C are a series of photographs showing the gelation of the collagen solution upon exposure to PBS. FIG. 4A shows the collagen solution prior to addition to buffer. FIG. 4B shows gelation of demeclocycline-collagen upon exposure to PBS. FIG. 4C shows the demeclocycline-collagen gel after one hour in buffer.

FIG. 5 is a chart showing cumulative fluorescamine release from

collagen gel over time.

FIG. 6 is a chart showing cumulative demeclocycline release from a collagen gel over time.

FIG. 7 is a graph describing the rate of fluorescamine release from a collagen gel. At 54 hours the percent release increased to 92.1%.

FIG. 8 is a graph describing the rate of demeclocycline release from a collagen gel.

FIG. 9 shows the spectrophotometric absorbance of acyclovir in saline.

FIG. 10 shows the spectrophotometric absorbance of a rabbit fibrillar collagen fraction crosslinked to acyclovir.

FIG. 11 shows the spectrophotometric absorbance of a human fibrillar collagen fraction (1:10 dilution) crosslinked to acyclovir. The analysis was conducted 8 days after treatment with 0.02% trypsin.

FIG. 12 shows the spectrophotometric absorbance of the first PBS wash (1:10 dilution) following trypsin treatment of the human fibrillar collagen fraction described in FIG. 11.

FIG. 13 shows the spectrophotometric absorbance of the second PBS wash following trypsin treatment of the human fibrillar collagen fraction described in FIG. 6.

FIG. 14 shows the spectrophotometric analysis of the human fibrillar fraction crosslinked to acyclovir following digestion with collagenase.

FIG. 15 shows the spectrophotometric analysis of an acyclovir-free human fibrillar fraction following digestion with collagenase.

FIG. 16 shows the spectrophotometric analysis of crosslinked acyclovir released from collagen-based gels into the supernatant from 1 to 48 hours.

FIG. 17 is a graph of crosslinked acyclovir released from collagen-based gels (absorbance) into the supernatant as a function of time.

FIG. 18 shows the spectrophotometric analysis of crosslinked acyclovir released from collagen-based film from 2 hours to 6 days.

FIG. 19 is a graph depicting the release of crosslinked acyclovir from collagen-based film into the supernatant as a function of time.

Detailed Description

Described herein is a delivery system which uses an initially soluble collagen capable of rapid polymerization as a vehicle for delivery of a therapeutic agent, for example, a cell preparation or therapeutic peptide. The low viscosity of the initial soluble collagen allows homogeneous mixing with the therapeutic agent and administration to a site, for example, by injection. The rapid polymerization characteristic of the collagen vehicle allows for targeted delivery and maintenance of the therapeutic agent at specific tissue sites. In addition, the dose and rate of delivery may be readily controlled by regulation of the concentration and *in vivo* release kinetics of the therapeutic agent.

Any collagen which exhibits the properties of an initially soluble state followed by rapid polymerization (preferably, within 180 seconds) may be used in the invention. Because this delivery system is optimally designed for use in mammalian systems, rapid polymerization preferably occurs at temperatures and pH's which approximate the physiological conditions of the recipient mammal. For humans, this collagen preferably polymerizes in a temperature range between 36°-39°C and at a pH range between 6.5-7.5.

Particularly preferred collagens for use in the invention are described in DeVore & Eiferman (U.S. Patent No. 5,492,135.) These collagens are

initially soluble in form and, upon exposure to physiological fluids *in vivo*, undergo rapid polymerization. Such collagen solutions have been prepared at concentrations ranging from 10 mg/ml to over 70 mg/ml and at pH's ranging from 6.0-8.0.

The collagen systems described herein may be used to deliver any therapeutic agent or pharmaceutical agent of appropriate solubility characteristics, including, without limitation, a cell preparation (wherein the cells themselves are therapeutic, e.g., a mesenchymal stem cell preparation or transfected cells which produce a therapeutic peptide), a growth factor, a steroid, an antibiotic, an angiogenic agent, an inhibitor of angiogenesis, and an inhibitor of cell proliferation. In each case, the therapeutic agent is added to the collagen solution to a desired concentration, and the solution is administered to the appropriate tissue site. The therapeutic agent can be entrapped in the collagen, or covalently bound and crosslinked to the collagen. Combinations of two or more therapeutic agents may be administered simultaneously, or the therapeutic agent may be combined with other compounds which enhance the therapeutic agent's effect. For example, a vasoactive compound, such as epinephrine, could be co-administered to restrict local blood flow, and, thus, reduce diffusion of the therapeutic agent away from the injection site.

If desired, the *in vivo* stability of the collagen formulation following injection can be increased by increasing its resistance to digestion and degradation. This is achieved by increasing the crosslinking in the collagen molecules by either adding a fibrillar component to the collagen solution or by chemically inducing a crosslinking reaction. A therapeutic agent can also be chemically bound and crosslinked to the collagen, in either soluble, partially soluble, or intact fibrillar form. Both collagen-collagen crosslinks and therapeutic agent-collagen crosslinks effectively slow the release of the

therapeutic agent, and, thus, extend the duration of delivery.

Any appropriate fibrillar component may be utilized to increase the *in vivo* stability of the collagen. For example, fibrillar collagen may be reconstituted collagen prepared from animal sources, such as bovine hide, using methods described in Borel and Randoux (Frontiers in Matrix Biology, Vol. 10, pages 1-58, In *Methods of Connective Tissue Research*, Eds. Robert, Moczar, and Moczar, S. Karger, Basel, 1985). Fibrillar collagen may also be prepared from human or animal tissue sources using methods described in Kelman and DeVore (U.S. Patent Nos. 4,969,912 and 5,322,802). The concentrations of the fibrillar collagen component may range between 0.1-2.0% fibrillar collagen solids (w/v).

There are many potential chemical "stabilizing" compounds for use in chemically cross-linking the collagen molecules and the therapeutic agents, e.g., crosslinking collagen amine groups together or crosslinking collagen amine groups to amine, carboxyl, phenol, sulfonyl, or carbohydrate groups of therapeutic agents. Suitable crosslinking compounds include bifunctional acylation agents, including anhydrides, acid chlorides, sulfonyl chlorides, e.g., 1,2,3,4-cyclobutanetetracarboxylic dianhydride, tetrahydrofuran-2,3,4,5-tetracarboxylic dianhydride, 1,2,4,5-benzenetetracarboxylic di-anhydride, ethylenediaminetetraacetic dianhydride, bicyclo(2,2,2)oct-7-ene-2,3,5,6-tetracarboxylic dianhydride, glutaryl dichloride, adipoyl chloride, 3-methyladipoyl chloride, pimeloyl chloride, terephthaloyl chloride, isophthaloyol dichloride, phthaloyl dichloride, 1,4-phenylene bis(chloroformate), 2,4-mesitylenedisulfonyl chloride, 2,6-naphthalenedisulfonyl chloride, malonyl dichloride, homobifunctional amine cross-reactive cross-linkers, such as homobifunctional imidoesters and homobifunctional N-hydroxysuccinimidyl, and heterobifunctional crosslinkers

(Pierce, Rockford, IL).

The collagen-therapeutic agent delivery system described herein is ideally suited to the treatment of hard tissue defects, including bone and cartilage repair, and soft tissue defects, including tendon, ligament, and dermal repair. Such defects can result from injury, degeneration, or from disease. The therapeutic agents administered to enhance repair include the following: for bone repair, mesenchymal stem cells, osteoprogenitor cells, and growth factors which either stimulate bone synthesis or inhibit its resorption, e.g., basic and acidic fibroblast growth factor, insulin-like growth factors I and II, and transforming growth factor beta; for cartilage repair, mesenchymal stem cells, chondrocytes, growth factors, cytokines, and angiogenic factors; for tendon and ligament repair, mesenchymal stem cells; and for general soft tissue repair and wound healing (e.g., in connective tissue, neural tissue, organs, or vasculature tissue), a promoter of fibroblast infiltration, a fibroblast cell preparation, and a promoter of collagen secretion, angiogenesis, or wound closure (e.g., PDGF-A, PDGF-B, FGF, VEGF, and leptin.) The collagen delivery system of the present invention may also be used for periodontal and anti-inflammatory treatments.

In another application, the collagen-therapeutic agent delivery system described herein is also suited to administer a therapeutic agent to inhibit cell proliferation. Such inhibition is beneficial, for example, in the treatment of cancer. The therapeutic agent is administered at the tumor or at the site of tumor resection to inhibit cancer cell proliferation.

Examples of cell proliferation inhibitors that are used as therapeutic agents are as follows: anti-metabolic antibiotics, e.g., mitomycin, daunorubicin, mithramycin, bleomycin, or doxorubicin; anti-metabolites, e.g., 5-fluorouracil, 5-fluorouridine-5'-monophosphate, 5-fluorodeoxyuridine, 5-fluorodeoxyuridine-5'-monophosphate, and 5-fluoroorotate; anti-fibrotics, such

as inhibitors of prolyl hydroxylase and lysyl hydroxylase (e.g., iron chelators, α,α-dipyridyl, o-phenanthroline, proline analogs, lysine analogs, and free radical inhibitors and scavengers), inhibitors of collagen secretion (e.g., colchicine, vinblastin, cytochalasin B, copper, zinc, and EGTA), inhibitors of collagen secretion and maturation (e.g., BAPN, vincristine, and D-penicillamine); stimulators of collagen degradation, e.g., EDTA and colchicine; anti-viral compounds, e.g., vidarabine, acyclovir, AZT, and amantadine; angiostatic compounds, e.g., angiostatin; and other miscellaneous anti-cell proliferative drugs, e.g., tissue plasminogen activator (TPA), heparin, cytosine arabinoside, and gamma-interferon.

In addition, it may be desirable to administer a collagen-antibiotic solution either as the sole therapeutic agent in patients at risk of developing local infection, e.g., post-operative and diabetic patients, or in combination with another therapeutic agent. Similarly, an antiviral compound can be administered, e.g., injection of a collagen-acyclovir formulation could provide a sustained release system for treating local outbreaks of herpes infection.

There is described below one set of experiments demonstrating that the present collagen delivery system provides a means by which cells may be delivered, for example, to a bone, cartilage, meniscus, tendon, ligament, or dermal defect, to promote healing through the stimulation of new matrix formation.

An additional set of experiments demonstrates that the present collagen delivery system may be used to deliver various therapeutic agents. The characteristics of the collagen formulation can be modified to allow for extending the duration of therapeutic agent release, if desired, by increasing the crosslinking within the collagen formulation and by crosslinking the therapeutic agent to the collagen.

These examples are provided for the purpose of illustrating the invention and should not be construed as limiting.

A Rapidly Polymerizing Collagen-Mesenchymal Stem Cell Preparation

Samples of collagen were prepared from rabbit dermis by the methods described in DeVore and Eiferman (U.S. Patent No. 5,492,135). The soluble collagen, formulated at either 10 or 20 mg/ml, was then mixed with 7.5 million rabbit mesenchymal stem cells (MSC). The collagen-MSC solution was then either (1) incubated for three weeks, during which time gelation occurred spontaneously, (2) polymerized to form a gel, incubated for 7 days, and then implanted into nude mice subcutaneously, or (3) immediately injected into nude mice subcutaneously. All implants were removed 3-6 weeks post-implantation and all collagen-MSC gels were examined histologically.

Collagen-MSC gels incubated *in vitro* for 7 days showed no matrix formation (Figs. 1A-B). In contrast, collagen-MSC gels removed from the mice demonstrated chondrocyte-like cell and metachromatic matrix formation, whether the gels were pre-polymerized and implanted, or polymerized after injection into mice. In pre-polymerized gels, chondrocyte hypertrophy and endochondral bone formation were observed after removal 3 weeks post-implantation (Figs. 2A-B). In gels polymerized after injection, an even more robust chondrogenesis and osteogenesis response was observed after removal 6 weeks post-injection. These gels also exhibited the formation of openings resembling marrow space (Fig. 3).

These results clearly demonstrated the ability of the rapidly polymerizing collagen formulation to deliver mesenchymal stem cells for enhancement of new matrix formation. In addition, all injection sites "set-up" into gelatinous-fibrous plugs with no indication of diffusion or migration.

These results indicated that this system is ideal for injectable delivery of mesenchymal stem cells to treat bone, cartilage, tendon, and ligament defects, as well as the delivery of other therapeutic agents to other specific sites.

Binding and Release of a Therapeutic Agent from a Collagen Preparation

Release of demeclocycline and fluorescamine from collagen gels

Samples of collagen were prepared from rabbit dermis by the methods described in DeVore and Eiferman (U.S. Patent No. 5,492,135). Briefly, type I collagen was extracted from calf hide, purified, and acid solubilized in 0.1N acetic acid, dialyzed extensively, and the pH adjusted to 7.5. To prepare the therapeutic agent-collagen samples, fluorescamine (1 mg/ml) or demeclocycline (15 mg/ml), in combination with a 5% coupling agent, was added to the collagen preparation and allowed to react (two hours for fluorescamine or 30 minutes for demeclocycline). The fluorescamine-collagen solution was then dialyzed against deionized water for 24 hours.

Both the fluorescamine- and demeclocycline-collagen solutions were formed rapidly into gels upon exposure to phospate buffered saline (Fig. 4). The gel pellets were gently agitated in buffer at 37°C. Aliquots of buffer were removed at various time points up to 54 hours, showing increasing concentrations of collagen and either fluorescamine (Fig. 5) or demeclocycline (Fig. 6), indicating that the agents were released as the pellets dissolved in the buffer.

These results demonstrate that the rapidly gelling collagen preparations provide a unique vehicle for local delivery of a therapeutic agent. The rapidly gelling aspect allows the collagen to set-up in a particular injection site and release the therapeutic agent at the site over a period of time. The duration of release may be a function of gel dissolution (Figs. 7 and 8).

Release of acyclovir from collagen gels, collagen films, and fibrillar collagen preparations

To assay the binding and release of acyclovir from various collagen preparations, rabbit or human dermis was minced, stirred in 70% alcohol for at least 2 hours, stirred in 0.1 N hydrochloric acid for at least 2 hours, and then submersed in 0.5 M Tris buffer, pH 9.0, for at least 2 more hours. The tissue was homogenized several times using a Waring blender, and acyclovir (Zovirax, 10 mg/ml, Burroughs Wellcome Co., Research Triangle Park, NC) was added. To this tissue homogenate, the bifunctional anhydride 3,3',4,4'-benzophene tetracarboxylic dianhydride (1:20 parts tissue) was added, followed by dispersion using glutaric anhydride (1:10 parts tissue). The tissue suspension was re-homogenized. After 30 minutes, an additional aliquot of glutaric anhydride (1:10 parts tissue) was added, and the tissue suspension was homogenized.

Following centrifugation (8,000 rpm for 20 minutes), the treated tissue suspension separated into a clear, gelatinous collagen fraction, a soluble collagen fraction, a fraction of fine, dispersed tissue containing fibrillar collagen, and particulate residue. The soluble fraction and the dispersed fibrillar collagen fractions were washed and stored in 70% alcohol. The gelatinous fraction was placed in saline solution (0.9%).

The collagen fractions were analyzed spectrophotometrically to determine acyclovir binding. Peak absorbance for acyclovir occurs at about 250 nM and 270 nM (Fig. 9). Acyclovir was present in the rabbit (Fig. 10) and human (Fig. 11) fibrillar collagen fractions, demonstrating acyclovir binding. Acyclovir binding in the human fibrillar fraction was demonstrated 8 days after trypsin treatment. No acyclovir appeared in the trypsin supernatant. After 24

days of trypsin treatment, the human fibrillar fraction was washed in PBS. Figures 12 and 13 show that some acyclovir was released in the first and second PBS washes, respectively (with the 270 nM acyclovir peak being diluted in the second wash). The PBS-washed human fibrillar fraction was then digested with bacterial collagenase. As shown in Figure 14, acyclovir was still bound to the human fibrillar fraction following 24 days of trypsin treatment and was released upon collagenase digestion, showing a typical acyclovir absorbance profile. No acyclovir absorbance peak was present in the control, non-acyclovir treated fibrillar fraction, as shown in Figure 15.

The gelatinous acyclovir fraction was placed in saline solution (0.9%) and saline aliquots were scanned spectrophotometrically to determine release of acyclovir over time. Figures 16 and 17 show the release of acyclovir from the collagen-based gels from 1 to 48 hours. The results demonstrated that acyclovir was released continuously for at least 48 hours.

The soluble fraction was also formed into a collagen-acyclovir film. The collagen solution was pipetted into a ceramic mold and air dried in the laminar flow hood. After 24 hours, the film was subjected to ultraviolet irradiation (to crosslink the collagen molecules) in nitrogen for 20 minutes. The film was placed in 0.9% saline solution and incubated at 37°C. Aliquots were taken for spectrophotometric evaluation. Figures 18 and 19 demonstrate acyclovir binding and the slow release kinetics from the collagen-based films. After 6 days, the film was still intact.

These experiments demonstrate that acyclovir can be chemically bound to fibrillar, gelatinous, and soluble collagen, and released as the collagen is enzymatically digested, ranging in duration from less than 1 hour to more that 28 days. Presumably, the release of the therapeutic agent continues until the collagen is completely dissolved. Collagen digestion was most rapid in the

gel formulation (which contained no fibrillar component), intermediate in the film, and slowest in the fibrillar fraction. Therefore, given that the rate of collagen digestion and resorption can be somewhat controlled by the degree of crosslinking (acylation) in the collagen formulation, the rate of therapeutic agent release and the duration of delivery can also be controlled by choosing the appropriate collagen formulation to be used in the injectable delivery system.

Other Embodiments

Other embodiments are within the claims. All publications mentioned herein are hereby incorporated by reference.

What is claimed is:

Claims

1. A method for delivering a therapeutic agent to a tissue site, said method comprising:

(a) combining said therapeutic agent with a soluble collagen to form a therapeutic agent-collagen solution; and

(b) administering said solution to said tissue site, whereby, upon contact with said tissue, said solution is converted to a gel and said therapeutic agent is delivered to said site.

2. The method of claim 1, wherein said solution is administered by injection.

3. The method of claim 1, wherein said tissue site is bone, cartilage, meniscus, tendon, ligament, or dermis.

4. The method of claim 1, wherein said tissue site has a defect.

5. The method of claim 4, wherein said defect is treated by delivery of said therapeutic agent to said tissue site.

6. The method of claim 1, wherein, upon administration, said solution is converted to a gel within 180 seconds.

7. The method of claim 6, wherein, upon administration, said solution is converted to a gel within 120 seconds.

8. The method of claim 7, wherein, upon administration, said solution is converted to a gel within 90 seconds.

9. The method of claim 1, wherein said solution comprises a fibrillar component.

10. The method of claim 9, wherein said fibrillar component is at a concentration of between 0.01-2.0% fibrillar collagen solids (w/v).

11. The method of claim 1, wherein the duration of delivery of said therapeutic agent is extended by crosslinking said therapeutic agent to said collagen in said solution.

12. The method of claim 1, wherein the duration of delivery of said therapeutic agent is extended by increasing the crosslinking of said collagen in said solution.

13. The method of claim 12, wherein said crosslinking is increased by adding a fibrillar component to said solution.

14. The method of claim 11 or 12, wherein said crosslinking is chemically induced.

15. The method of claim 11 or 12, wherein said crosslinking is induced by UV radiation.

16. The method of claim 1, wherein said therapeutic agent is a

growth factor, a steroid, an antibiotic, an angiogenic agent, an inhibitor of angiogenesis, or an inhibitor of cell proliferation.

17. The method of claim 1, wherein said solution is at approximately pH 7.0.

18. A therapeutic agent-collagen solution, whereby, upon contact with a tissue, said solution is converted to a collagen gel.

19. The therapeutic agent-collagen solution of claim 18, wherein said therapeutic agent-collagen solution is injectable.

20. The therapeutic agent-collagen solution of claim 18, wherein said therapeutic agent-collagen solution is converted to a collagen gel within 180 seconds.

21. The therapeutic agent-collagen solution of claim 20, wherein said therapeutic agent-collagen solution is converted to a collagen gel within 120 seconds.

22. The therapeutic agent-collagen solution of claim 21, wherein said therapeutic agent-collagen solution is converted to a collagen gel within 90 seconds.

23. The therapeutic agent-collagen solution of claim 18, wherein said therapeutic agent-collagen solution further comprises a fibrillar component.

24. The therapeutic agent-collagen solution of claim 23, wherein said fibrillar component is at a concentration of between 0.01-2.0% fibrillar collagen solids (w/v).

25. The therapeutic agent-collagen solution of claim 18, wherein said therapeutic agent is crosslinked to said collagen in said solution.

26. The therapeutic agent-collagen solution of claim 18, wherein said collagen is crosslinked in said solution.

27. The therapeutic agent-collagen solution of claim 25 or 26, wherein said crosslinking is chemically induced.

28. The therapeutic agent-collagen solution of claim 25 or 26, wherein said crosslinking is induced by UV radiation.

29. The therapeutic agent-collagen solution of claim 18, wherein said therapeutic agent is a growth factor, a steroid, an antibiotic, an angiogenic agent, an inhibitor of angiogenesis, or an inhibitor of cell proliferation.

30. The therapeutic agent-collagen solution of claim 18, wherein said solution is at approximately pH 7.0.

31. A method for delivering cells to a tissue site, said method comprising:

(a) combining said cells with a soluble collagen to form a cell-collagen solution; and

(b) administering said solution to said tissue site, whereby, upon contact with said tissue, said solution is converted to a gel and said cells are delivered to said site.

32. The method of claim 31, wherein said cells are stem cells.

33. The method of claim 32, wherein said stem cells are mesenchymal stem cells.

34. The method of claim 31, wherein said solution is administered by injection.

35. The method of claim 31, wherein said tissue site is bone, cartilage, meniscus, tendon, ligament, or dermis.

36. The method of claim 31, wherein said tissue site has a defect.

37. The method of claim 31, wherein said defect is treated by delivery of said cells to said tissue site.

38. The method of claim 31, wherein, upon administration, said solution is converted to a gel within 180 seconds.

39. The method of claim 38, wherein, upon administration, said solution is converted to a gel within 120 seconds.

40. The method of claim 39, wherein, upon administration, said

solution is converted to a gel within 90 seconds.

41. The method of claim 31, wherein said solution comprises a fibrillar component.

42. The method of claim 41, wherein said fibrillar component is at a concentration of between 0.01-2.0% fibrillar collagen solids (w/v).

43. The method of claim 31, wherein the duration of delivery of said cells is extended by crosslinking said cells to said collagen in said solution.

44. The method of claim 31, wherein the duration of delivery of said cells is extended by increasing the crosslinking of said collagen in said solution.

45. The method of claim 44, wherein said crosslinking is increased by adding a fibrillar component to said solution.

46. The method of claim 43 or 44, wherein said crosslinking is chemically induced.

47. The method of claim 43 or 44, wherein said crosslinking is induced by UV radiation.

48. The method of claim 31, wherein said solution is at approximately pH 7.0.

49. A cell-collagen solution, whereby, upon contact with a tissue, said solution is converted to a collagen gel.

50. The cell-collagen solution of claim 49, wherein said cells are stem cells.

51. The cell-collagen solution of claim 50, wherein said stem cells are mesenchymal stem cells.

52. The cell-collagen solution of claim 49, wherein said cell-collagen solution is injectable.

53. The cell-collagen solution of claim 49, wherein said cell-collagen solution is converted to a collagen gel within 180 seconds.

54. The cell-collagen solution of claim 53, wherein said cell-collagen solution is converted to a collagen gel within 120 seconds.

55. The cell-collagen solution of claim 54, wherein said cell-collagen solution is converted to a collagen gel within 90 seconds.

56. The cell-collagen solution of claim 49, wherein said cell-collagen solution further comprises a fibrillar component.

57. The cell-collagen solution of claim 56, wherein said fibrillar component is at a concentration of between 0.01-2.0% fibrillar collagen solids (w/v).

58. The cell-collagen solution of claim 49, wherein said cells are crosslinked to said collagen in said solution.

59. The cell-collagen solution of claim 49, wherein said collagen is crosslinked in said solution.

60. The cell-collagen solution of claim 58 or 59, wherein said crosslinking is chemically induced.

61. The cell-collagen solution of claim 58 or 59, wherein said crosslinking is induced by UV radiation.

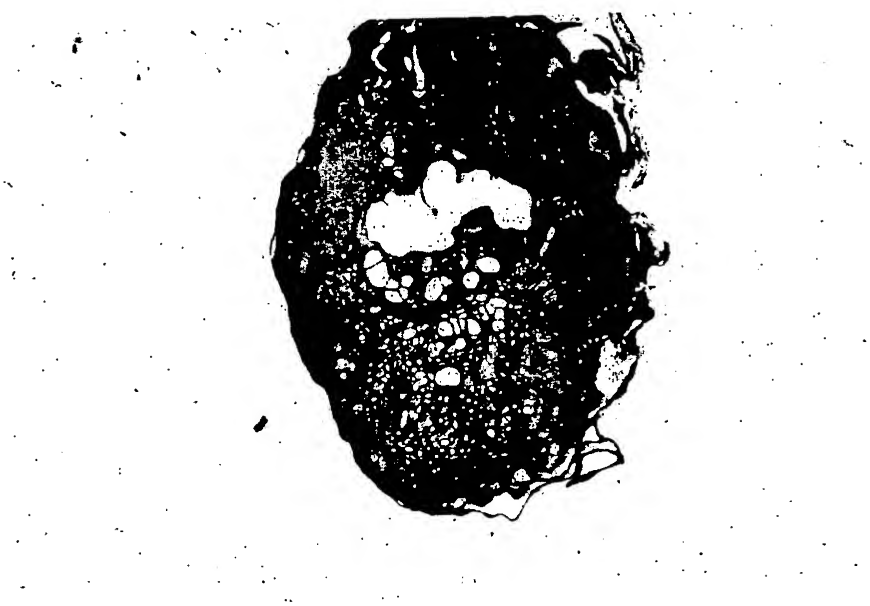
62. The cell-collagen solution of claim 49, wherein said solution is at approximately pH 7.0.

RPC seeded with Rabbit Mesenchymal stem cells (MSCs) 7 days in vitro

Fig.1A

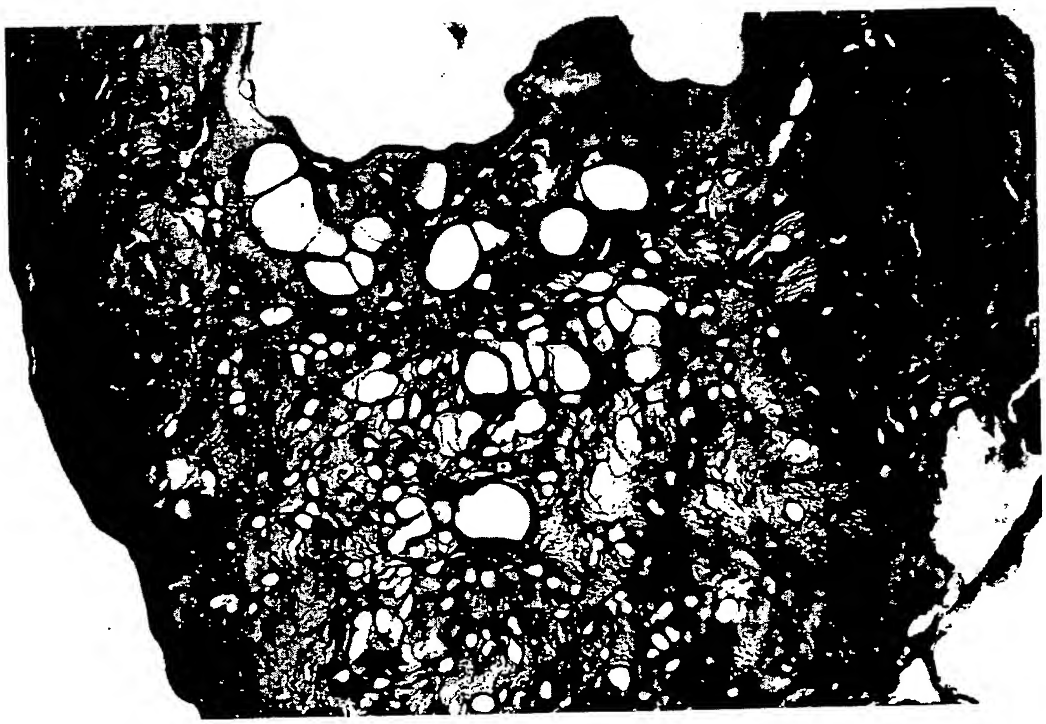
RPC seeded with Rabbit Mesenchymal stem cells (MSCs) 7 days in vitro

Fig.1B



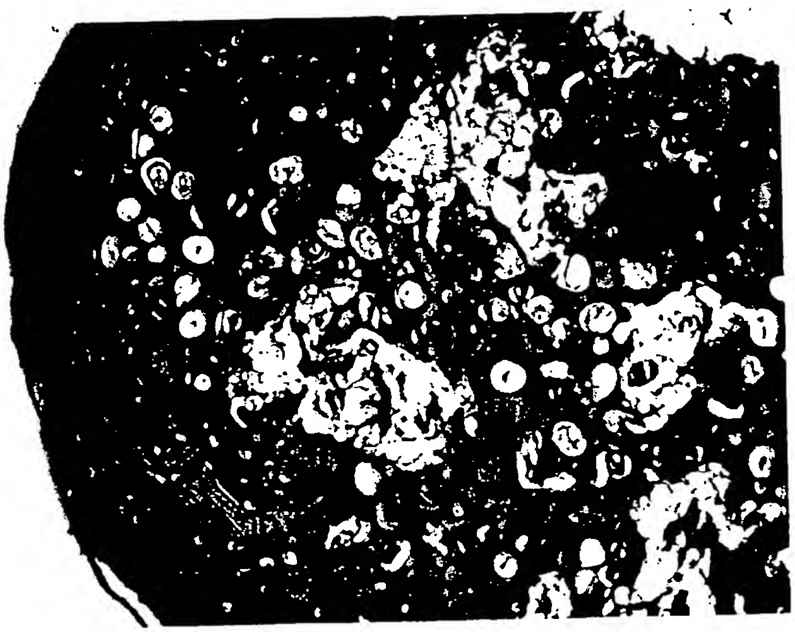
RPC seeded with Rabbit MSCs
3 weeks in vivo

Fig. 2A



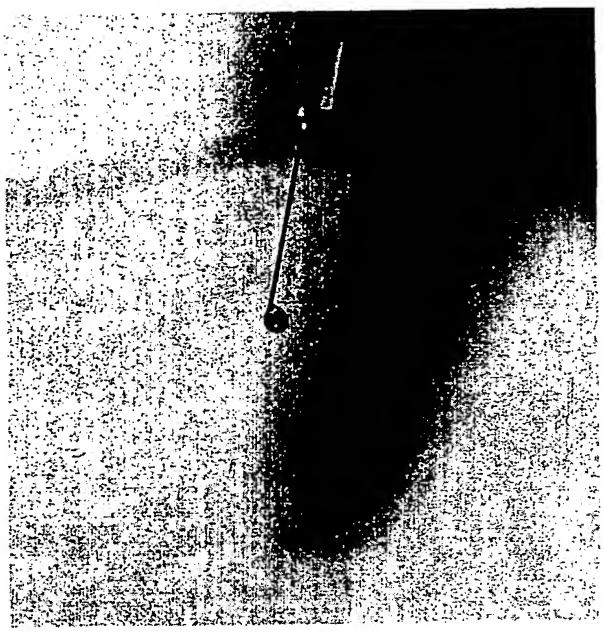
RPC seeded with Rabbit MSCs
3 weeks in vivo

Fig. 2B



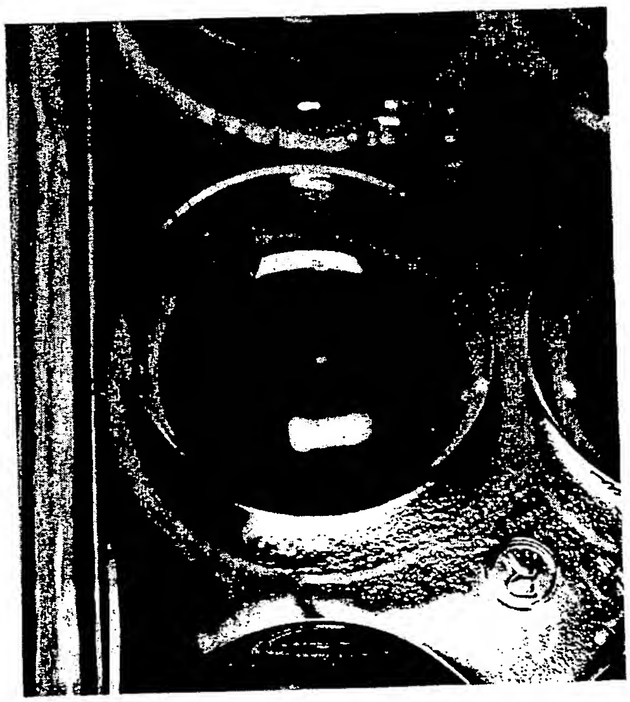
RPC seeded with Rabbit MSCs
6 weeks in vivo

Fig. 3



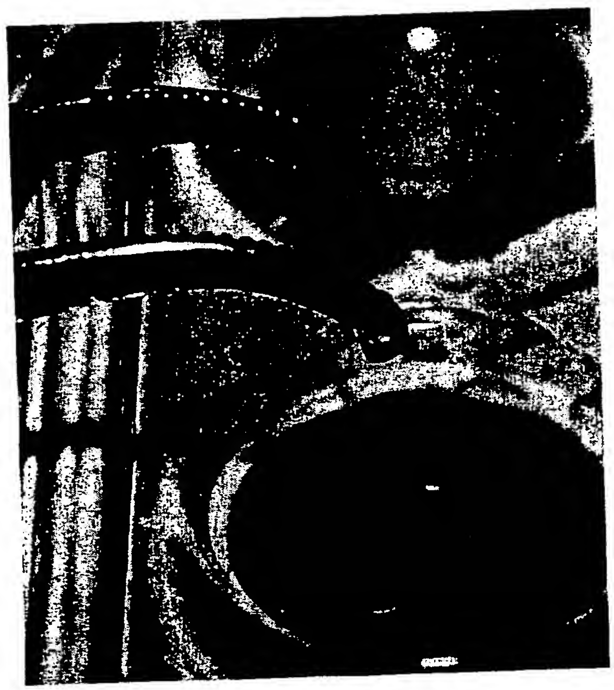
DROPLET OF DEMECLOCYCLINE-COLLAGEN SOLUTION PRIOR TO ADDITION TO BUFFER

Fig. 4A



GELATION OF DEMECLOCYCLINE-COLLAGEN

Fig. 4B



DEMECLOCYCLINE-COLLAGEN GEL AFTER ONE HOUR IN BUFFER

Fig. 4C

RELEASE OF FLUORESCAMINE FROM COLLAGEN GELS (CUMULATIVE)

TIME (HRS)	AMOUNT RELEASED (μg)	RELEASE PERIOD
1	18	LIMITED GEL DISSOLUTION
4	22	
6	84	RELEASE OF (F) DURING GEL DISSOLUTION
12	244	
24	340	

◇ RATE OF RELEASE FROM 0-4 HRS = 5.5μg/HR

◇ RATE OF RELEASE FROM 4-24 HRS = 15.9μg/HR

Fig. 5

DEMECLOCYCLINE RELEASE FROM COLLAGEN GELS (CUMULATIVE)

TIME (HRS)	AMOUNT RELEASED (MG)	RELEASE PERIOD
1	10.4	DIFFUSION OF UNBOUND (D)
4	10.7	
6	11.0	
12	11.6	STEADY-STATE RELEASE OF (D) DURING GEL DISSOLUTION
18	12.0	
24	12.7	

◇ RATE OF RELEASE FROM 1-24 HRS = 87μg/HR

Fig. 6

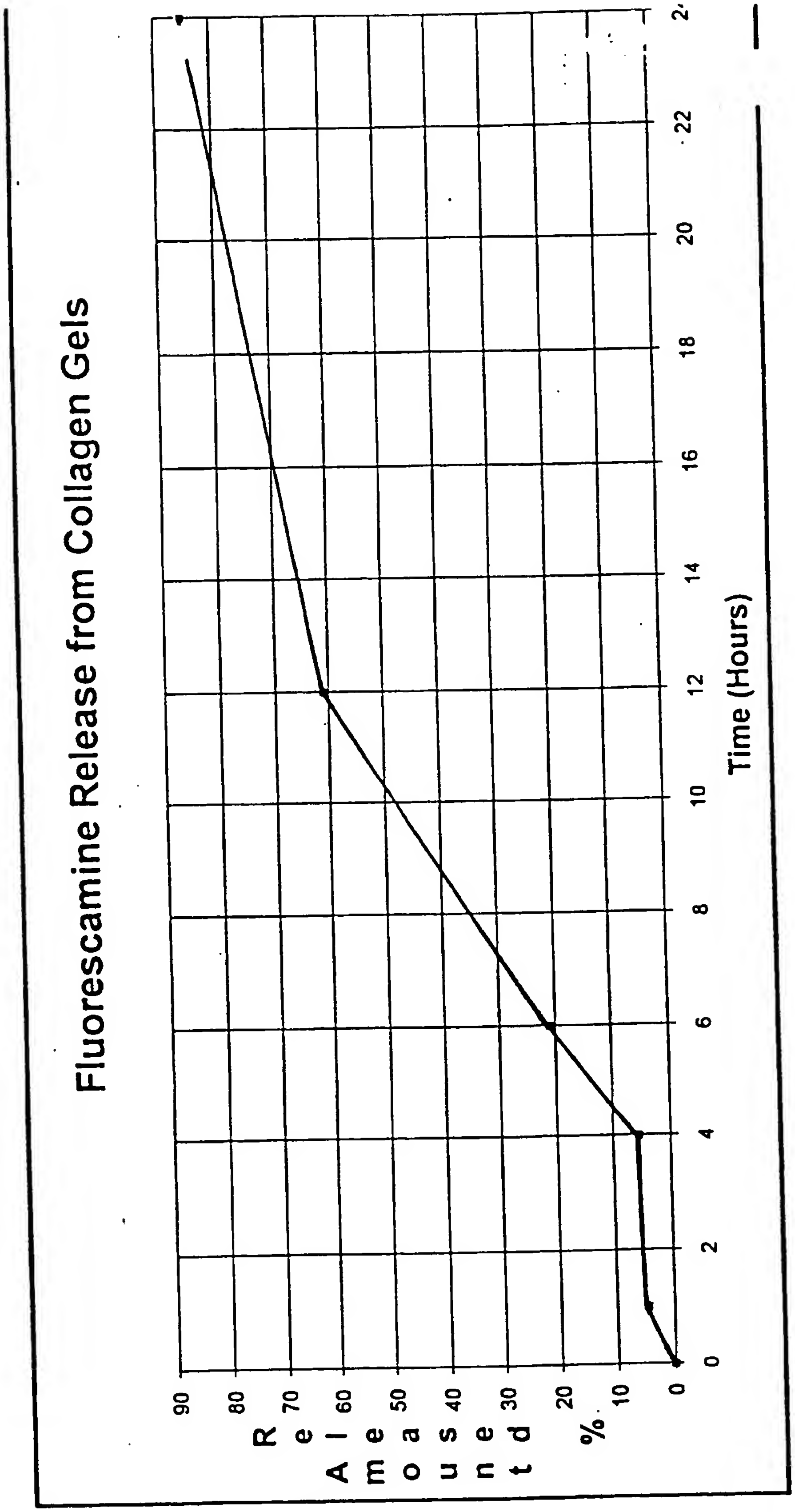

Fluorescamine Release from Collagen Gels
Time (Hours)
Released Amount %
0
2
4
6
8
10
12
14
16
18
20
22
0
10
20
30
40
50
60
70
80
90

Figure 7

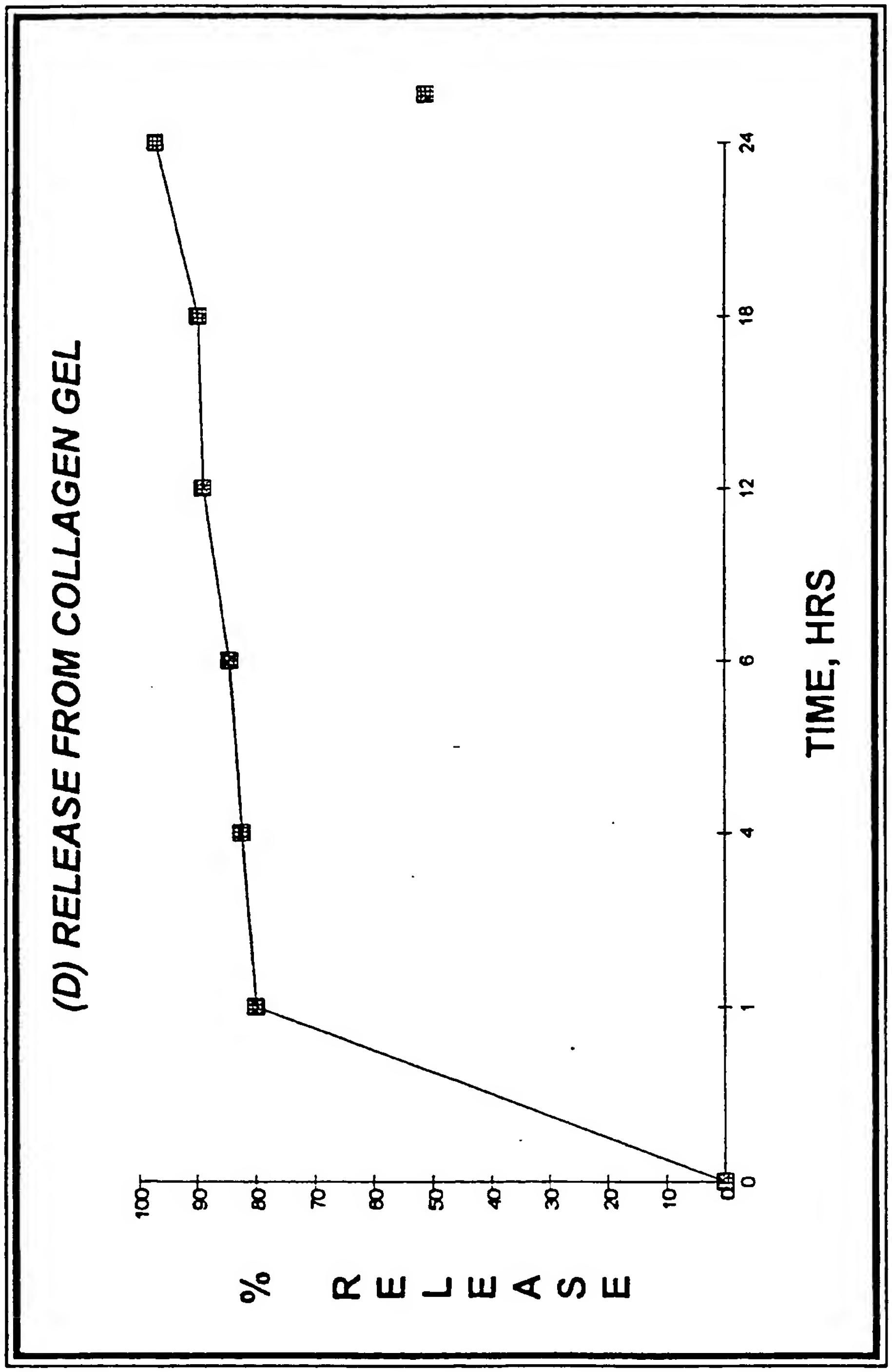
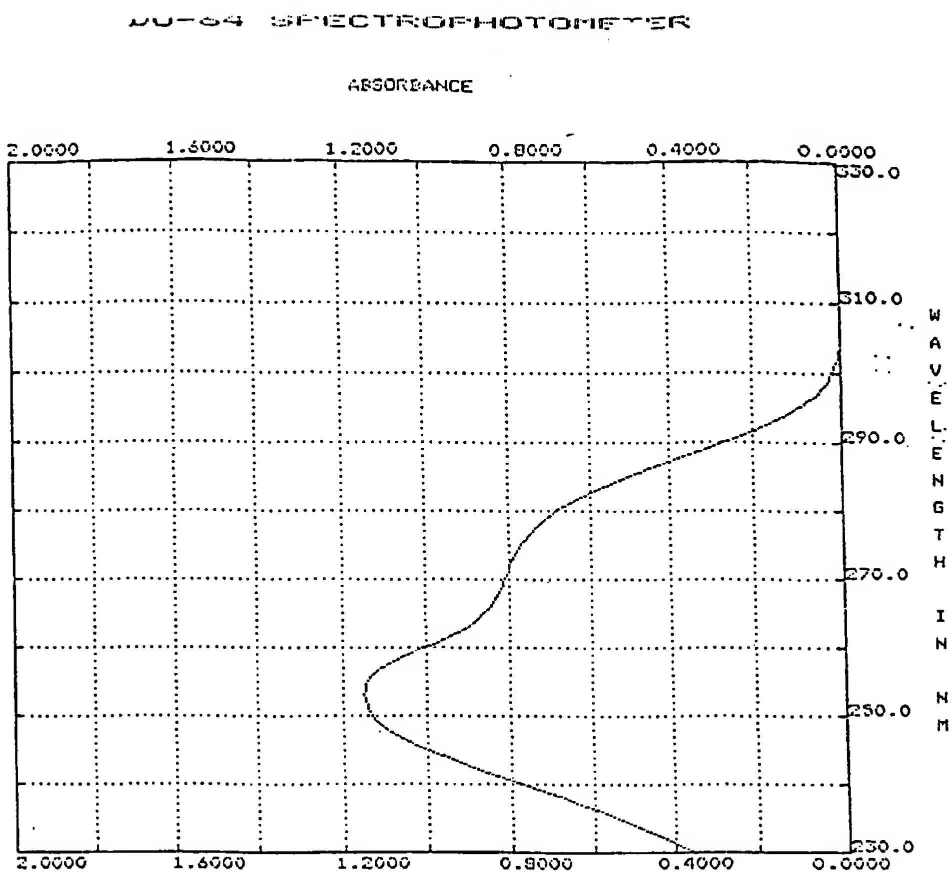

(D) RELEASE FROM COLLAGEN GEL
% RELEASE
100
90
80
70
60
50
40
30
20
10
0
0
1
4
6
12
18
24
TIME, HRS

Figure 8



Scan Speed: 750 nm/min

PEAK PICK

λ	Abs
325.0	0.000
253.0	1.148

POINT PICK

λ	Abs
280.0	0.633
270.0	0.811
260.0	1.008
250.0	1.132
240.0	0.772

Figure 9

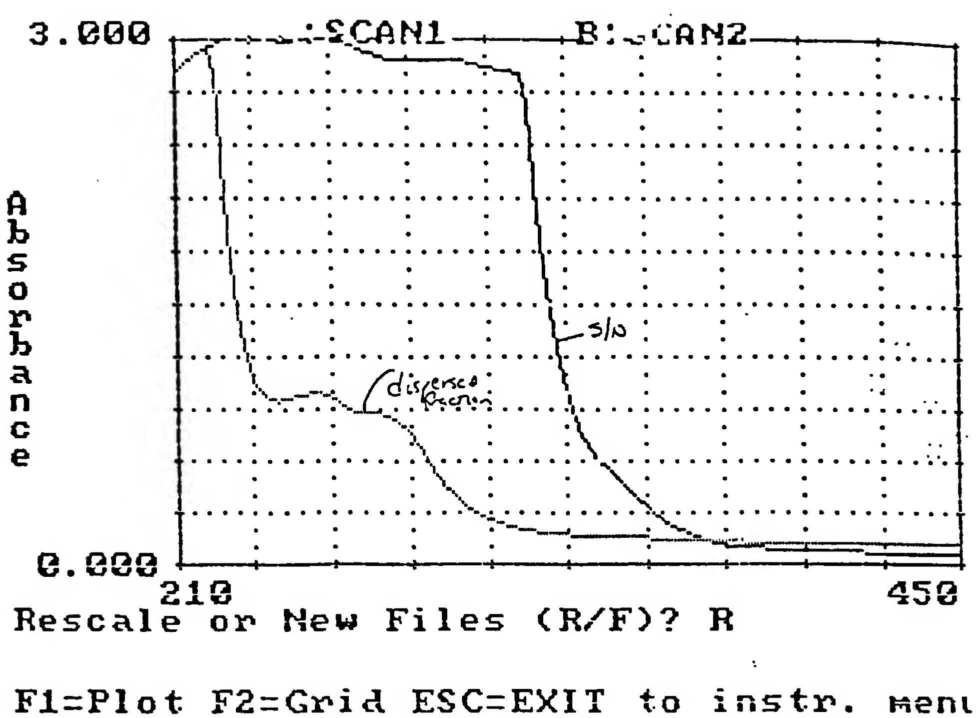
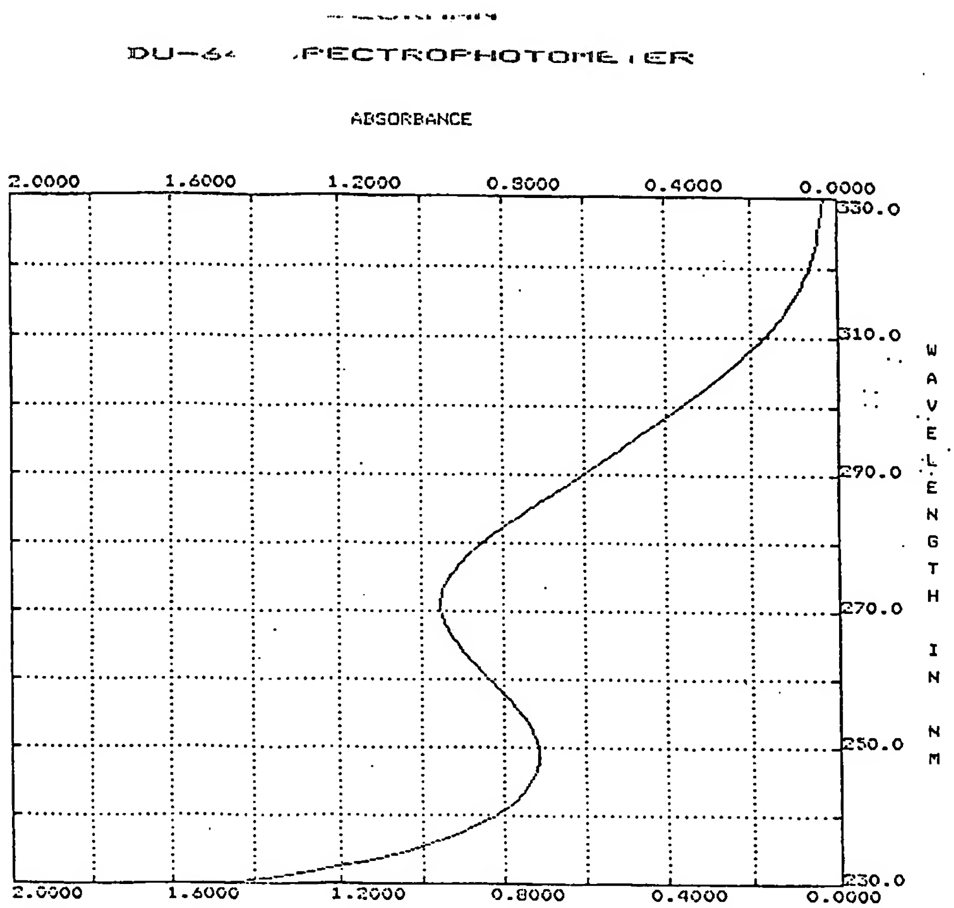

3.000
:SCAN1
B: CAN2
Absorbance
s/n
0.000
210
450
Rescale or New Files (R/F)? R
F1=Plot F2=Grid ESC=EXIT to instr. menu

Figure 10



Scan Speed: 750 nm/min

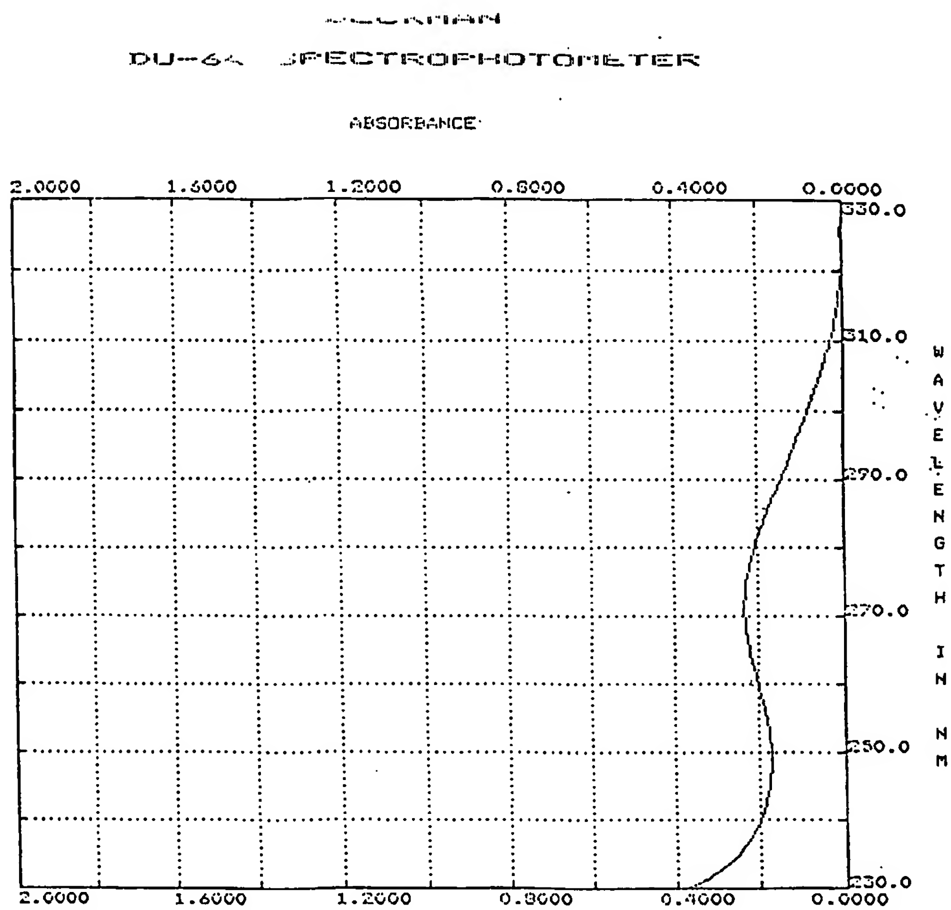
PEAK PICK

λ	Abs
271.0	0.955

POINT PICK

λ	Abs
270.0	0.953
265.0	0.911
260.0	0.836
250.0	0.717
240.0	0.822

Figure 11



Scan Speed: 750 nm/min

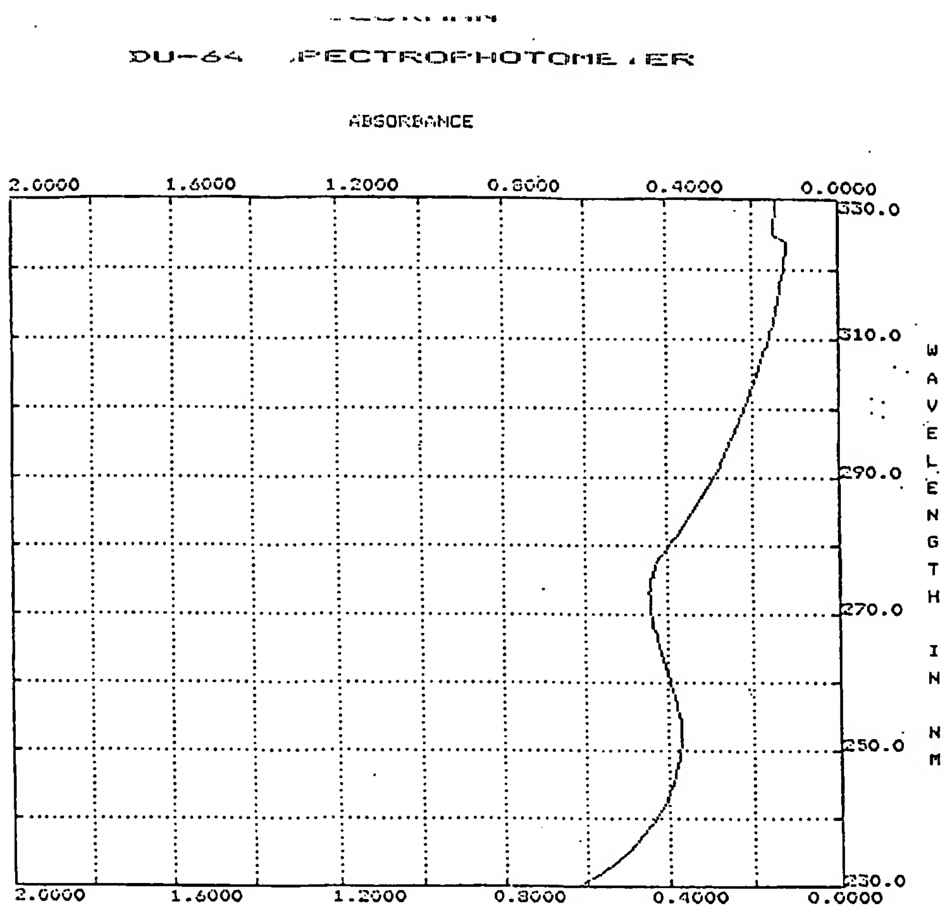
PEAK PICK

λ	Abs
325.0	0.005
271.0	0.234

POINT PICK

λ	Abs
270.0	0.233
265.0	0.221
260.0	0.202
250.0	0.172
240.0	0.198

Figure 12



Scan Speed: 750 nm/min

PEAK PICK

λ	Abs
325.0	0.151
273.0	0.443

POINT PICK

λ	Abs
270.0	0.438
265.0	0.420
260.0	0.396
250.0	0.373
240.0	0.431

Figure 13

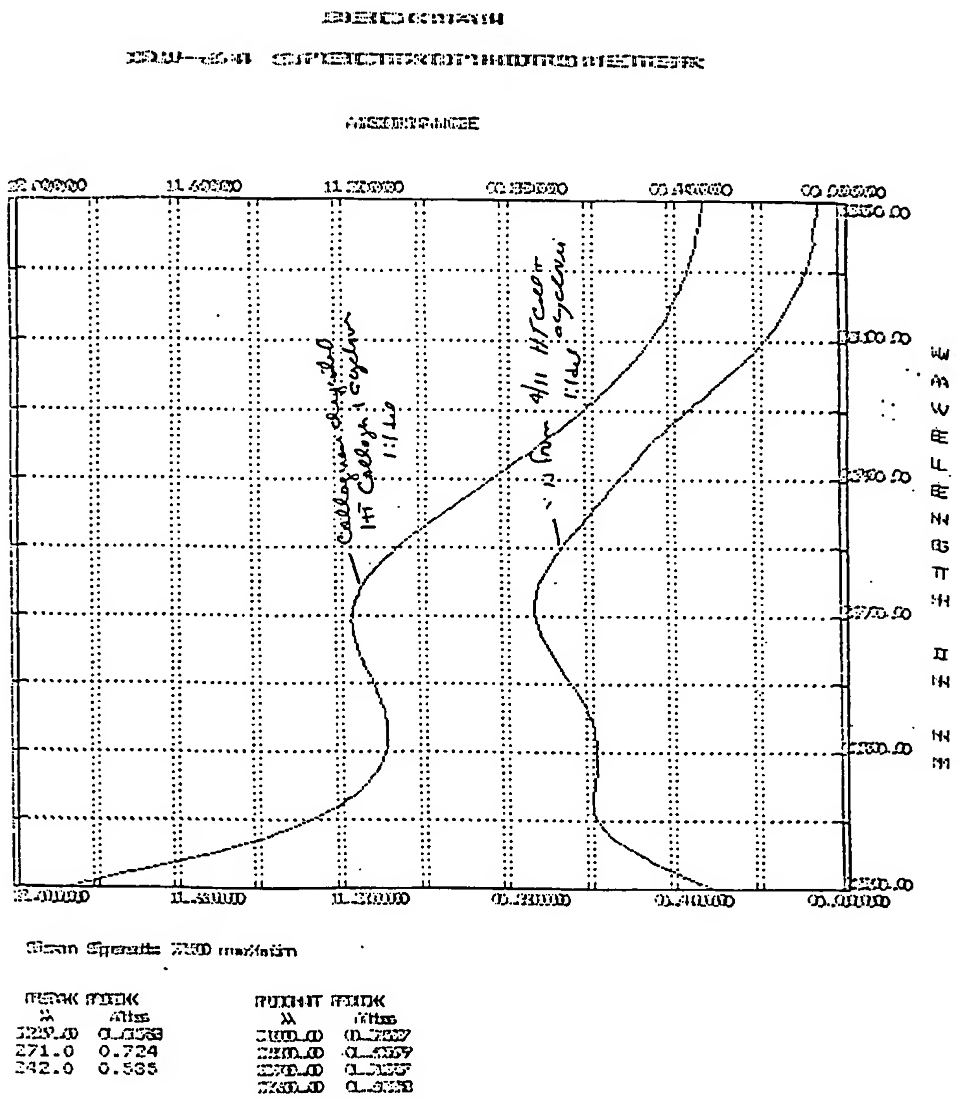
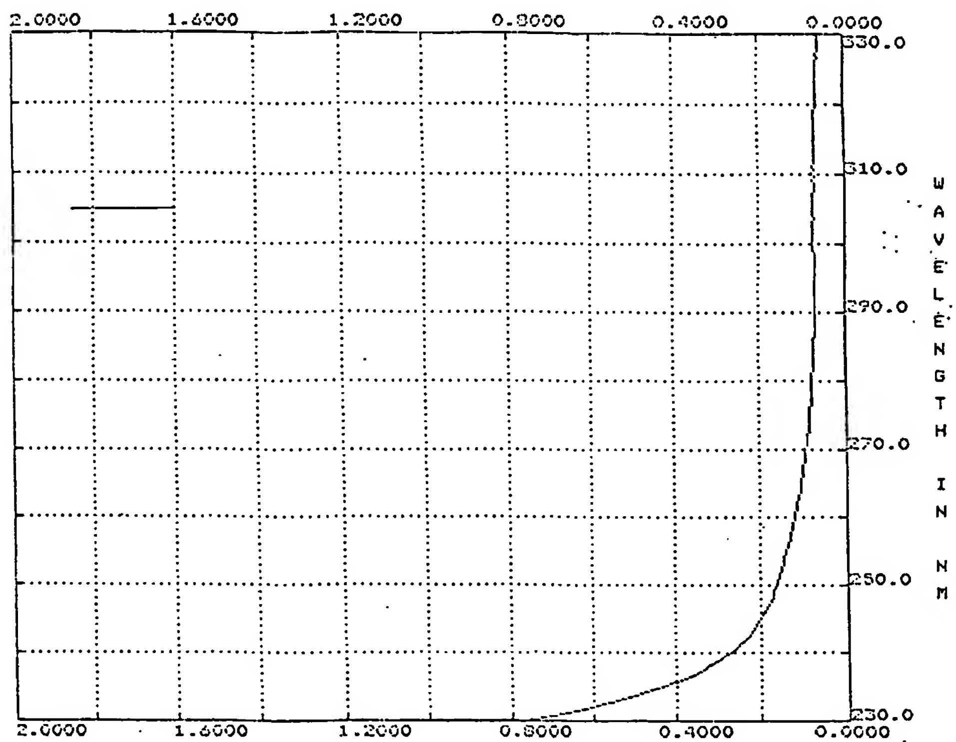


Figure 14

DU-64 . PECTROPHOTOME.ER

ABSORBANCE



Scan Speed: 750 nm/min

PEAK PICK

λ	Abs
309.0	0.073
305.0	0.074
300.0	0.074
290.0	0.070

POINT PICK

λ	Abs
300.0	0.074
280.0	0.078
270.0	0.093
260.0	0.120
265.0	0.106

Figure 15

18/21

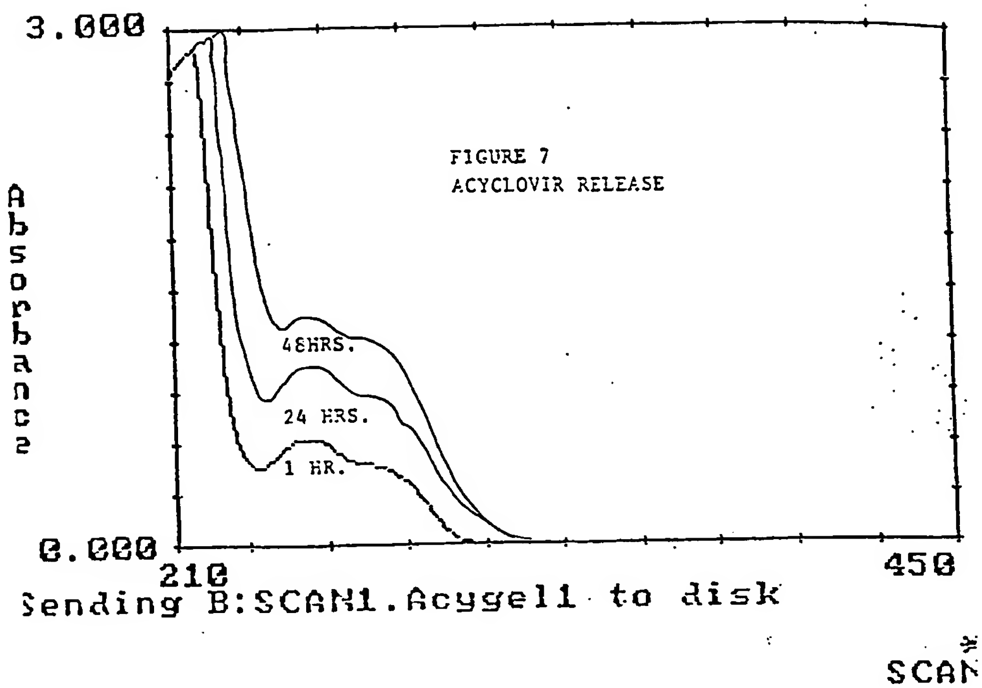
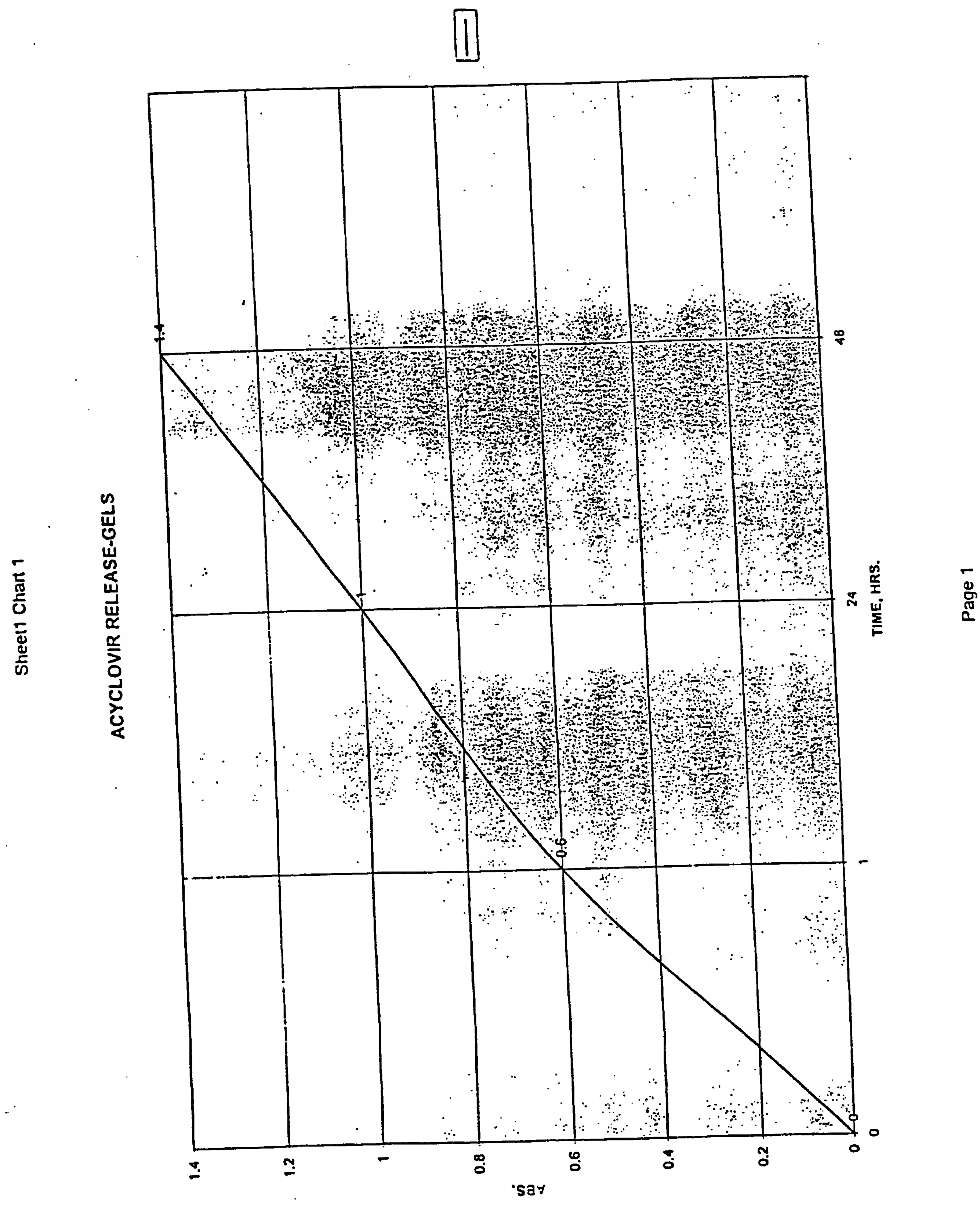


Figure 16

Figure 17


Sheet1 Chart 1
ACYCLOVIR RELEASE-GELS
ABS.
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TIME, HRS.
0
1
24
48
Page 1

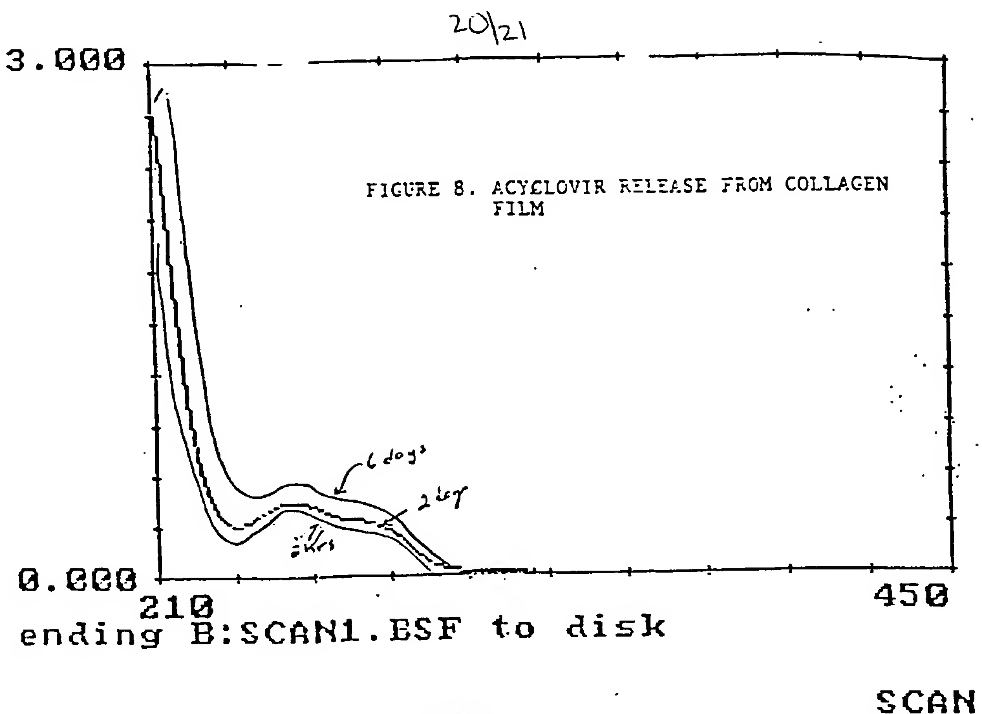

20/21
3.000
FIGURE 8. ACYCLOVIR RELEASE FROM COLLAGEN FILM
6 days
2 day
0.000
210
450
ending B:SCAN1.BSF to disk
SCAN

Figure 18

Sheet1

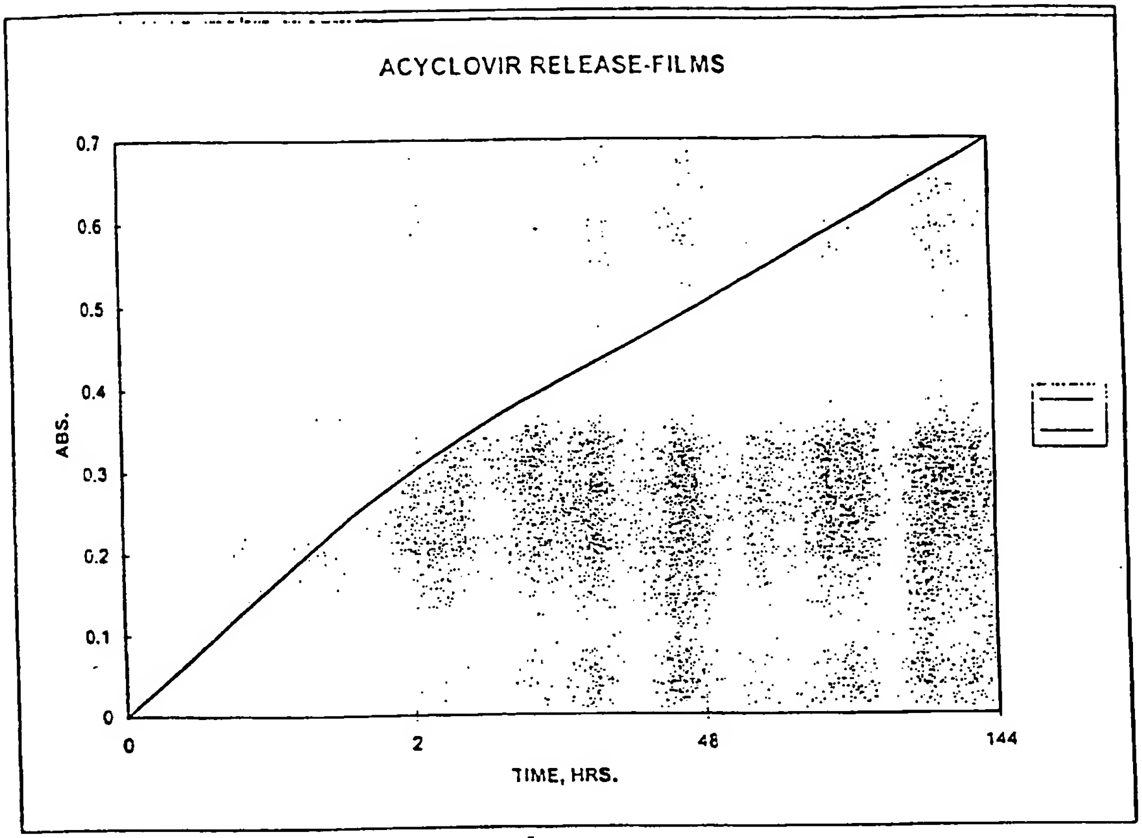


Figure 19

INTERNATIONAL SEARCH REPORT

International application No. PCT/US00/03117

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61F 2/02
US CL : 424/426
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/426

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,660,692 A (NESBURN et al) 26 August 1997, see entire document.	1-62
Y	US 5,550,188 A (RHEE et al) 27 August 1996, see entire document.	1-62

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search: 08 MAY 2000

Date of mailing of the international search report: 25 MAY 2000

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Commissioner of Patents and Trademarks
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